DETERMINING THE EFFECT OF ARSENIC ON THE IMMUNE SYSTEM DURING PREGNANCY AND INFLUENZA RISK

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ABSTRACT

Problem Statement: Pregnancy is a critical window of susceptibility for mothers due to impaired function of immunity. During the second trimester, the balance between T helper cell subtypes (Th1 and Th2) is altered and a shift towards a Th2 immune response reduces a pregnant mothers' ability to fight against viral infections, including influenza A virus (IAV). Environmental factors such as inorganic arsenic (iAS) exposure can also have a significant impact on the immune response against pathogens. Populations living in iAs exposed areas may have increased risk of viral infection and related morbidity due to the detrimental health effects from iAs exposure. However, there is limited evidence examining the relationship between arsenic exposure and the risk of influenza in pregnant mothers. This study aimed to address this knowledge gap by determining the effect of iAs exposure on the immunity of pregnant mothers against IAV infection.

Methods: Pregnant C57BL/6 mice were treated with 100ppb of inorganic arsenic for 5 weeks prior to being infected with IAV (10 $TCID_{50}$). The effect of iAs exposure on the immune response during IAV infection in pregnant mice was assessed by changes in (1) lung viral titers measured by a Median Tissue Culture Infectious Dose ($TCID_{50}$) assay; (2) the innate and adaptive cytokine/chemokine response quantified using a 32-multiplex Luminex assay; and (3) the innate and adaptive immune cell numbers and functions were analyzed by flow cytometry.

Result: Chronic iAs exposure significantly reduced alveolar macrophages (AMs) numbers in mediastinal lymph nodes (MLNs) (*p* value = 0.0421), but did not significantly increase viral titer, alter cytokine and chemokine responses or other immune cell numbers and activation in the

lungs or MLNs.

Conclusion: Our findings suggest that chronic iAs exposure affects immune responses against IAV infection by reducing AMs numbers during pregnancy. Future studies should consider repeating this study with increased sample size and a higher viral infection dose to confirm these conclusions. Addressing these limitations in future studies will offer valuable insights into the relationship between arsenic exposure and IAV infection during pregnancy.

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INTRODUCTION

Immune responses against influenza infection

An estimated 300,000 to 600,000 global deaths per year can be attributed to influenza infections [1]. In the United States (U.S.), influenza infections are responsible for approximately 200,000 hospitalizations and 41,000 deaths annually. Moreover, it is the 7th leading cause of death in the country [2]. Influenza is a contagious respiratory disease caused by the influenza virus. This disease primarily affects the upper respiratory organs including the nose, throat, bronchi, and lungs [3]. Experts believe that the respiratory droplets from infected individuals can spread to others up to 6 feet away via common behaviors such as talking, sneezing, or coughing [4]. More importantly, individuals with influenza infection can be asymptomatic, but their potential to pass the virus to others is not diminished. Typically, influenza infections result in mild symptoms such as fever and cough. However, a severe immune response and secondary infections, such as pneumonia, are also common in influenza patients [1, 2]. In fact, pregnant women have a higher chance of developing severe illness, classifying this group as a vulnerable population. Even more, a study on the 2009 Influenza A Virus (IAV) epidemic in the U.S. found that pregnant women were also at higher risk of mortality [5], potentially due to changes in the immune system during pregnancy [6]. Therefore, the general public, particularly pregnant women, should not overlook the severity of influenza infection.

Influenza viruses are enveloped, single-stranded RNA viruses categorized into four main types: A, B, C, and D [7-9]. Only influenza A viruses (IAV) are known to cause global epidemics with flu-like symptoms being the main clinical outcome [10]. IAV can escape the first line of the

defense—physical barriers and can survive the innate immune response [11]. On the surface of the virus, hemagglutinin (HA) is the most abundant glycoprotein [12]. HA enables the virus to enter host cells. Due to HA's high variability, neutralizing antibodies produced by B cells are not capable of properly recognizing the virus resulting in widespread IAV infection [11]. IAV mostly infects the airways and alveolar epithelial cells [13-16]. Therefore, IAV infections are almost always associated with respiratory tract damage, facilitating the onset of respiratory diseases such as pneumonia [17].

Despite viral evasion, the immune system uses its defense to ameliorate the situation. One essential part of the innate immune response is the immediate action from different phagocytic cells. First, the viral compounds are recognized as pathogen associated molecule patterns (PAMPs) by the pathogen recognition receptors (PRRs) on the host [18, 19]. This will initiate signal transduction for cytokine and antiviral production through PRRs. The nuclear factor-κB (NF-κB) pathway is also crucial for pro-inflammatory cytokine production, such as tumor necrosis factor (TNF), whereas antiviral responses are induced by type I interferons (IFN) [20]. The presence of tumor necrosis factor-alpha (TNF- α) and interleukin-1 (IL-1) can trigger innate immune cells to migrate to the site of infection and amplify the signal enough to not only induce phagocytic cell signaling, but also an adaptive immune response [21]. Innate immune cells, such as NK cells can kill virus-infected cells whereas neutrophils can phagocytize apoptotic virus-infected cells. Dendritic cells (DCs)—also known as professional antigen-presenting cells (APCs)—can also phagocytize virus-infected cells and present immune peptides (antigens) to activate an adaptive immune response [21]. When lung epithelial cells are infected by IAV, conventional DCs (cDCs) migrate to the lymph node to activate naïve T cells and B cells [22-24].

Depending on which major histocompatibility complex (MHCI or MHCII) is bound, naïve T cells will either be activated as IAV-specific CD8+ cytotoxic T cell (CTL) or CD4+ T helper (Th) cells, respectively [24]. B cells can then be activated by CD4+ Th cells and subsequently become plasmablasts to produce IAV-specific antibodies. T cells require 3 elements to reach their most efficient potential: cytokine presence, antigen presentation, and a co-stimulatory signal [25]. For example, IL-12 helps to activate CTLs; APC, including DCs, are responsible for cross-presentation of exogenous antigen through the MHC I complex; and B7 ligands on APC co-stimulate CTLs activation [26-30]. CTLs can kill virus-infected cells by forming pores in the target cell membrane with perforin and inducing apoptosis with granzyme [21].

Influenza risk in pregnant women

During pregnancy, a mother's immunity is not static and highly varies throughout the different stages of development. In humans, during the first 12 weeks of pregnancy, immune cells gather and initially cause inflammation at the lining of the womb to ensure the development of the fetus. However, the immune response is later downregulated for the following 15 weeks to allow the growth of fetal cells [31]. Essentially, the development process consists of three stages of immunological events [32]. First, implantation and placentation involve a pro-inflammatory stage in which tissue injury and repair are continuous. Associated cytokines such as interleukin-6 (IL-6), IL-8, IL-15, and TNF are produced by endometrial stromal cells and infiltrate the immune system during this stage [33]. Secondly, there is an anti-inflammatory stage that occurs during fetal growth. This process incorporates immune cells such as T regulatory (T_{reg}) cells, decidual NK cells, and macrophages to maintain an anti-inflammatory environment [34]. The decidual macrophages secrete anti-inflammatory cytokines

predominantly at this time [35]. Lastly, another pro-inflammatory stage occurs in preparation for labor where the NF-κB pathway is crucial for labor initiation [36]. Also, both IL-1 and TNF-α are viewed as central players during this final stage [37]. Hence, pregnant mothers are usually more susceptible to pathogens because their immune system is altered during pregnancy [38].

Many immune mechanisms involving NF-κB and TNF-α are also altered to maintain pregnancy. For instance, studies suggest that NF-κB activity is suppressed until labor during pregnancy [36, 39]. However, NF-κB is also important for DC development, a key component for activating T cells to respond to IAV infections [40]. Reduced NF-κB activity can be detrimental for CTLs responses to IAV infections. Data also exist suggesting that TNF-α can induce preterm labor in pregnant animals [37]. However, one of the functions of TNF-α is to activate macrophages, the first main line of defense against IAV infection [41]. It is hypothesized that reduced TNF-α levels may affect immunity against IAV infections through insufficient macrophage response. Because many immune mechanisms are altered throughout pregnancy, it is still unknown which altered immune responses are responsible for increased susceptibility to pathogens in pregnant mothers.

Similar to humans, female mice also experience unstable immunity during pregnancy [42]. One of the major differences is that pregnancy in mouse models is shorter--a normal pregnancy averaging 19-20 days. This short timeframe elicits a more rapid immune response that parallels that of humans despite its short duration. Likewise, in order to successfully carry the fetus through pregnancy, maternal immune tolerance also exists in pregnant mice [42]. One noteworthy change in immunity is a shift between Th1/Th2 immune response in both human

and mice during pregnancy [43, 44]. Hence, while ensuring a successful pregnancy, pregnant mothers are simultaneously risking the integrity of their own immunity during this time.

Compromised immunity during pregnancy leads to increased vulnerability to IAV infections in both humans and mice, but how maternal immunity is altered during pregnancy remains controversial [45]. Nevertheless, one of the leading factors affecting immune-responses is hormones [46]. Besides being the two key sex steroid hormones responsible for maintaining the progression of pregnancy, progesterone and estrogen are also associated with immune responses against IAV infection; however, their role during the immune response is not well characterized [47, 48]. During pregnancy, estradiol, the most abundant estrogen subtype, is one of three different estrogens that can be produced by the mother and the placenta [49, 50]. Interestingly, in mouse studies focused on viral infection in pregnant mice, estradiol was found to reduce the severity of the disease [51-53]. Pazos et al. found a significant reduction in the innate immune response against influenza infection in both pregnant mice and estradiol-treated non-pregnant mice. Specifically, decreased levels of IFNγ, keratinocyte chemoattractant, and TNF-α, delayed viral clearance, and reduced CD8+ T cell activity was also observed [51]. Therefore, the authors concluded that estrogen may be protective for fetal development by impairing immune responses. Conversely, despite the findings on anti-inflammatory responses, chronic administration of estradiol was recognized to promote Th1 cytokine production in murine microglial cells and DCs [54-56], as well as enhancing the inflammatory response initiated by macrophages [57]. These two distinctive effects on immune responses suggest that estrogen has a significant influence on immune responses, and the effects may vary as the level of estrogens fluctuate throughout pregnancy.

Like estrogen, progesterone is also produced by the corpus luteum and placenta during pregnancy. Progesterone primarily modulates the mother's immune response to prevent embryo rejection and suppresses uterine contractions [58]. In general, progesterone inhibits inflammatory innate immune responses [48]. For example, progesterone produced by the placenta has been associated with the downregulation of macrophage responses and NK cell activity [59]. Additionally, *in vitro* studies have established that progesterone promotes T cells to shift more towards a Th2 response, with increased production of anti-inflammatory cytokines including IL-4, IL-5 and IL-10 [60-62]. By inhibiting Th1 and promoting Th2 immune responses, progesterone primarily increases the production of IL-4 and IL-5 [63]. Furthermore, in progesterone-treated ovariectomized mice, researchers found an earlier disease onset along with increased morbidity and mortality after influenza infection [64]. It was suggested that progesterone-treated mice possibly had dominant anti-inflammatory responses (driven by IL-10) that could promote worse disease outcomes. These changes in immunologic status would, therefore, increase the mother's susceptibility to infections [38].

Sex hormones oscillate during pregnancy, and their influence on immune responses against IAV infections vary because their concentrations change as pregnancy progresses. Based on the discoveries mentioned above, it is known that progesterone can reduce immune responses, but it is unclear how estrogen mediates immune responses. Therefore, it's difficult to identify the pathways that affect post-IAV infection during pregnancy.

Environmental factors and health

Hazardous substances commonly found within households and occupational hazards are a few of several environmental factors that have been implicated to have harmful effects on

immunity. According to the Office of Disease Prevention and Health Promotion, maintaining a healthy environment is important for human health [65]. Consequently, individuals adversely affected by environmental factors would display compromised immune function and poor immune status which would result in greater susceptibility to numerous illnesses.

Despite living in modern times, exposure to hazardous chemical substances is still prevalent in many locations across the globe [66]. Because these environmental toxicants are ubiquitous and have widespread use, it is not surprising that individuals are highly exposed to these chemicals. These toxicants originate from a variety of sources, including solvents in building material and plasticizers in food packaging. Plasticizers, for example, provide us with versatile products that are useful for handling different kinds of food packaging or making water pipes in diverse angles or lengths. Nevertheless, the material itself may be toxic to human health [67]. For instance, toxicity studies and human exposure data suggest that plasticizers may be a threat to human fertility and reproduction [68-70]. In animal studies, plasticizers were found to have adverse effects on fertility and reproduction in rats as well [71]. Also, animal studies indicate that chronic exposure to phthalates (the main chemical component in plasticizers) leads to low semen quality [72]. It is likely that these harmful effects may also occur in humans [73]. Although it may be possible to avoid exposure to these health-damaging substances by stopping the use of products that contain them, other environmental toxicants are far more difficult to avoid. One group of environmental toxicants that render major concern is heavy metals [74].

Heavy metals and immunotoxic effects

Heavy metals are metallic elements that have a higher density than water [75]. Although heavy metals are naturally occurring substances that exist in the earth's crust, human health and environmental quality are at high stake of being affected. Due to anthropogenic and natural activities, heavy metals can be released from the earth's crust, most commonly through groundwater [76]. Hence, humans and environmental sources are easily exposed to heavy metals dissolved in groundwater. Without proper water treatment and monitoring systems, human and environmental health are greatly impacted, especially in regions that rely on groundwater as their main water source. With regard to human health, more and more studies have demonstrated that heavy metals affect an individual's immunity significantly [77, 78], and some of these heavy metal exposures are still prevalent particularly in developing countries [79].

Lead (Pb)

Since the early 1900s, Pb has been widely used in water pipes. The physical and chemical properties of lead make water pipes resistant to pinhole leaks [80]. Unfortunately, once the pipes are damaged, Pb will be released into the water, which results in health problems, such as hypertension and renal dysfunction [81]. In an occupational exposure study, lead exposure was associated with inhibited lymphocyte proliferation [82]. Several animal studies demonstrate that Pb exposure results in dysregulated T cell responses specifically related to the production of helper cell subtypes. After Pb exposures, naïve T cells are skewed toward the Th2 subtype, which propagates the induction of Th2 cell development and proliferation [83], whereasTh1 development was inhibited by Pb through elevated adenylate cyclase activity [84]. In a study

conducted by Hsia et al., schoolchildren with low Pb exposure had increased Th2 immune response, which may contribute to respiratory allergic disorders [85]. These findings suggest that Pb related health problems may result from imbalanced Th1/Th2 responses.

Cadmium (Cd)

Cadmium can be released into the environment through mining, and it is found to affect human health by entering the food chain [86]. It has been documented that cadmium exposure via inhalation during childhood is associated with lung cancer in adulthood [87]. Not surprisingly, children with elevated urinary cadmium levels also had suppressed immune function. These effects are not limited to humans. According to Turley et al., a low level of chronic cadmium exposure induced IFNγ and IL-10 production in rats [88]. Since IL-10 can be produced by different T cell subsets and inhibits IFNγ expression, this suggests that a low level of chronic cadmium exposure may have impacts on more than one T cell subset.

Arsenic (As)

The majority of inorganic arsenic (iAs) exposures can be attributed to occupational exposures, but exposure via contaminated air, water, and food is also possible [89]. Symptoms associated with acute iAs exposure include nausea, vomiting, and diarrhea, whereas chronic exposures result in skin lesions and increased risk of cancers (bladder, kidney, lung) [90]. Results from a human study of Bangladeshi adults with skin lesions indicate that those with chronic iAs exposure have a higher concentration of serum total Immunoglobulin G(IgG), total IgE, and total IgA compared to the controls [91]. Immunoglobulins (also known as antibodies) are vital for their roles in protecting the host from pathogens [92]. Likewise, in arsenic-exposed children in rural Bangladesh, arsenic exposure positively correlated with total levels of IgG and IgE [93].

IgG and IgE protect the lower respiratory tract against infections [94] and serve as the major mediator of asthma [95], respectively. Even though the mechanism of how arsenic elevates the concentration of immunoglobins is unclear, the positive association between arsenic exposure and plasma IgG shows that arsenic exposure interferes with the integrity of the immune system. Also, a study on low levels of arsenic exposure found that pro-inflammatory cytokines such as IL-6, IL-8, and TNF-α have increased expression in exposed women [96]. The researchers suggest that this may increase a female's susceptibility to chronic inflammatory diseases in the long run.

Arsenic Compounds

Arsenic is a natural element embedded in the earth's crust [97]. It consists of both inorganic and organic forms. The inorganic form along with its metabolites is extremely hazardous to the environment and human health. In contrast to organic arsenic compounds, iAs is simple in molecular structure, but highly toxic [98]. In its oxidation state, inorganic arsenic (As^{3+}) can strongly bind with functional groups (e.g. thiolates of cysteine residues), and thereby inactivate proteins that depend on binding to cysteine thiols to function [99]. Over the past two decades, arsenic exposure through drinking water and soil has become a global concern due to its impact on public health [100, 101].

Genotoxic carcinogen

Even though human studies may not show a dose-response effect [102], *in vitro* studies on the effects of iAs on human lymphocytes and fibroblasts indicate that iAs is a genotoxic carcinogen [103-105]. With increased concern regarding the human health effects from iAs, more and more regulatory agencies around the world have revised the safety limit for iAs exposure. For

instance, in 2001 the U.S. Environmental Protection Agency, or EPA, replaced the previous standard (50ppb) with one that was five times less (10ppb) [106]. This change arose after the careful evaluation of multiple studies investigating the health effects of arsenic as well as expert opinions from renowned groups such as the National Research Council (NRC), the National Drinking Water Council, and the Science Advisory Board. According to the NRC estimation, a lifetime exposure of 10ppb iAs can result in a cancer incidence of 1 out of 300. This signifies that individuals with low exposure can still have a high cancer risk. Most importantly, carcinogens do not have thresholds [107], which means that carcinogens are harmful to health at any given level. It is crucial to understand that there is no safe level of arsenic exposure and even low arsenic exposure has elevated risks of developing diseases [108]. Yet, despite this, federal agencies continue to keep 10ppb as the safe drinking water standard for arsenic because many of the public water systems in the U.S. cannot afford to comply below this standard [109].

Metabolic pathway

iAs exists in two forms—inorganic arsenate (iAs^{5+}) and inorganic arsenite (iAs^{3+}). To date, how iAs is metabolized in our body remains controversial [110]. Generally, it is acknowledged that when iAs is absorbed into the body, iAs^{5+} needs to be reduced to iAs^{3+} before proceeding into further steps in the metabolism pathway [111]. Once it is reduced by glutathione (GSH), iAs^{3+} is methylated by arsenic (+3 oxidation state) methyltransferase (AS3MT) with S-adenosylmethionine (SAM) serving as a methyl donor, which then converts iAs^{3+} to pentavalent monomethylarsonic acid (MMA^{5+}). Next, MMA^{5+} is reduced to MMA^{3+} by GSH, followed by another round of methylation that generates dimethylarsinic acid (DMA^{5+}). Finally, DMA^{5+} is

reduced to dimethylarsinous acid (DMA^{3+}) by GSH [112]. In humans, the majority of iAs is excreted in the urine as dimethylarsinate (DMA) [113-116] (Figure 1).

Different metabolites between humans and rodents

Although the genetic resemblance between mice and humans is highly similar [117], iAs metabolism is not identical. Mice are believed to have an extra methylation step in their iAs metabolic pathway easing the formation of additional iAs metabolites other than MAs and DMAs which are generated after 1st and 2nd methylation. Trimethylarsine oxide (TMAO), for example, is formed after the methylation of DMA^{3+} (Figure 1). In humans, the presence of TMAO in urine is extremely low due to AS3MT's poor efficiency in methylating DMA^{5+}. Therefore, it is important to be mindful of the differences in the arsenic metabolic pathway between mice and humans when translating arsenic-related findings from one species to the other.

Methylation

In mammals, methylation of arsenic is considered an important process for iAs excretion [118]. This process decreases iAs' reactivity with cells and makes it more readily excreted [118, 119]. This explains why many iAs exposure-related health problems are associated with methylation events. Arsenic is associated with one of the best known and most common examples of epigenetic gene regulation, DNA methylation [120]. Epigenetic regulation is defined as altering gene expression without modifying the DNA sequence [121]. Interestingly, both DNA methylation and iAs methylation steps require SAM as a methyl donor, thus the reason why problems occur with iAs exposure. iAs exposures may absorb the majority of SAM within the body, and interfere with other cellular methylation reactions, including DNA methylation.

Therefore, environmental factors like heavy metals, can influence epigenetic programming that alters gene expression and eventually leads to diseases such as cancer, hypertension, and Type 2 diabetes [122].

Aside from epigenetic changes, polymorphisms in the human *AS3MT* gene are also known to be related to insufficient arsenic methylation, resulting in arsenic-related diseases [123]. According to a study on indigenous women living in northern Argentina, single nucleotide polymorphisms (SNPs) in the *AS3MT* gene accounted for a large portion of the variation of arsenic metabolism observed between individuals, especially the second methylation step [124]. It was revealed that individuals with specific SNPs have higher concentrations of monomethylsarsenic (MAs) in their urine, meaning that the second methylation step is compromised. Furthermore, there was an increased prevalence of skin lesions and DNA damage in these individuals [125, 126]. These observations suggest that the risk of iAs related health problems is disproportionately distributed based on direct genetic modifications and indirect epigenetic changes.

Alternative pathway for arsenic metabolism

Methylated oxyarsenicals are the typical byproduct post arsenic methylation. However, a study on sodium arsenate metabolism by anaerobic microbiota in mouse cecum discovered six other thioarsenical metabolites, including mono-, di-, and trithio-arsenic acid, monomethyldithio- and monomethyltrithio-arsonic acid, and dimethyldithioarsonic acid [127]. Thioarsenicals share a similar structure with oxyarsenicals with the main difference being that sulfur from hydrogen sulfide (H_2S) replaces oxygen on oxyarsenicals. In *in vitro* studies on the mouse cecum, it was demonstrated that the organisms that produce both an H_2S anaerobic microbiota and high pH in the environment induce the production of thioarsenicals. Once either iAs^{3+} or iAs^{5+} is

absorbed into the body, they become potential substrates for the conversion from oxyarsenicals to thioarsenicals. However, only iAs^{3+}-containing oxyarsenicals can proceed to the common oxyarsenical methylation pathway [127]. One study showed that the metabolites of thioarsenicals were found to have more cytotoxic effects on human uroepithelial EJ-1 cells [128]. This may imply that the presence of H_2S-producing anaerobic microbiota may increase arsenic toxicity within organisms. In a human study on arsenic exposed women in Bangladesh, thio-dimethylarsinate was present in 44% of the urine samples collected, ranging from trace amount to 24µg As/L (one sample contained up to 123µg As/L) [129], which means thioarsenicals may also be important for iAs metabolic pathways. Overall, these findings suggest that iAs metabolism may be more complex than originally proposed.

Arsenic Affected Organs

iAs is a human carcinogen that targets multiple organs including the skin, lungs, urinary bladder, and the liver [130]. Studies have reported an association between arsenic exposure and basal cell carcinomas or squamous cell carcinomas [131]. In an epidemiological study conducted on residents living in Hungary, Romania, and Slovakia, researchers found that the risk of basal cell carcinoma development was closely related to low-level arsenic exposure (≤100µg/L inorganic arsenic) [132]. In another study in Taiwan, an association between arsenic exposure and bladder cancer was discovered [133]. The average incidence of bladder cancer in endemic regions in Taiwan is roughly 5 times higher than their neighboring endemic areas [133]. However, this interpretation was based on a considerably high level of arsenic exposure (>0.35ppm) [134]. More studies are needed to not only identify the level of arsenic exposure that leads to bladder cancer, but to also emphasize the impact of confounding factors, such as

smoking [135]. A 20-year retrospective cohort study conducted on patients with liver cancer found an increased incidence in areas with arsenic levels above 0.64mg/L [136]. Whether arsenic is a hepatocarcinogen remains controversial. Multiple studies were conducted to determine the relationship between arsenic exposure and human liver cancer, and no general agreement could be made [137, 138]. On the other hand, some studies suggest lung cancer and arsenic exposure may have a dose-dependent relationship [139]. This relationship is best exemplified in Taiwan where there is an increased mortality rate of lung cancer among patients consuming water with high levels of arsenic contamination [140].

Like humans, iAs has similar effects on multiple organs in rodents. Studies have found that exposure to ingested iAs results in lung tumors in mice and urinary bladder tumors in rats [141, 142]. Similarities between humans and rodents result in the widespread use of rodents as the gold standard to study the effect of arsenic on organs and other tissues [143]. However, there are some differences between the two species, and translating findings between rodents and humans may require more comprehensive data. For instance, mice with 2 years of 50ppb of arsenic exposure had a higher incidence of bronchioloalveolar tumors [144]. But arsenic levels in groundwater are usually lower than 10ppb, and regions with high levels of arsenic exposures are mostly around 1ppm [145]. These findings suggest that rodents are more resistant to arsenic effects than humans, and it is necessary to develop a human-relative dose of arsenic exposure when translating findings from rodents to humans.

Effects of arsenic on influenza infection

Whether the influenza virus can successfully infect and replicate within the host is largely dependent on the host immune system [146]. Specifically, the virus-specific adaptive immune

response is responsible for the final control of viral replication [147]. This reaction includes antibodies, CD4+ T cells, and CD8+ T cells. Antibodies neutralize viral proteins and participate in viral clearance along with other immune cells [148]; CD4+ T cells stimulate B cells and antibody production, as well as activation and proliferation of CD8+ T cells [149]; CD8+ T cells are responsible for clearing the virus through cytolysis and producing cytokines that aid inflammatory responses against the virus [150]. Therefore, how arsenic affects influenza infection within the host may largely be due to its influence on those cells.

In general, iAs is viewed as an immunomodulatory agent [151-155]. When determining the effect of a moderate dose (100ppb) of iAs on IAV infection in a mouse model, researchers found that arsenic-treated male mice had an increased cell percentage and total cell number of CD8+ T cells relative to non-exposed groups, whereas no difference was observed in CD4+ T cells between the groups at 7 days post-infection (p.i.) [156]. Additionally, the absolute number of lymphocytes wasn't affected. It was suggested that the increased number of CD8+ T cells in the arsenic-treated group reduced the ability to clear the virus. Cytokine levels (IL-1β, IL-6, MIP-1α, RANTES, MCP-1, IL-10, M-CSF, MIP-2, MIP-1β, and TNFα) were also examined and despite no significant changes in cytokine levels at 7 days p.i., arsenic-treated mice had at least twice as many more cells in the lung than the non-exposed mice. The authors propose that cytokine production may still be reduced on a per-cell basis.

In another mouse study that focused on *in utero* and postnatal arsenic exposure, researchers discovered that the mice exposed to 100ppb arsenic had significantly higher viral titer at 7 days p.i. compared to the controls. There was also a significant increase in the number of neutrophils in the bronchoalveolar lavage fluid (BALF) samples that were collected from exposed mice at 7

days p.i.. This suggests that developmental exposure to arsenic may worsen inflammatory responses against early life IAV infections [157].

In children, arsenic was found to increase granulocyte-macrophage colony-stimulating factor (GM-CSF) while also reducing all of the following: peripheral blood mononuclear cells (PBMCs) IL-2 secretion and proliferation, CD4+ T cell count and the CD4+/CD8+ T cell ratio without altered CD8+ cell population [158]. Increased GM-CSF may indicate the presence of chronic inflammation [159], whereas reduced CD4+/CD8+ may allude to immunosuppression [160, 161], which can undermine immune responses against viral infection because CD4+ T cells are important for virus-specific CD8+ T cell activation [150].

Additionally, arsenic exposures may also exhibit different effects based on sex. For example, Vegal et al. found that T lymphocytes and PBMCs from women with a high dose of arsenic exposure showed more reduced cell proliferation than their arsenic exposed male counterparts [162]. Specifically, they found that arsenic was more toxic for CD4+ T cells than CD8+ T cells in women. CD4+ T cells were significantly reduced at 1 uM of sodium arsenite exposure, while CD8+ T cells were not affected [162]. These findings highlight how arsenic exposure leads to varied immune responses among men and women.

Based on available data, it is acknowledged that arsenic exposure worsens influenza infection outcomes. However, findings on T cell responses are not consistent across different species. According to Kozul et al., there is no difference in cell count changes in CD4+ T cells and an increased number of CD8+ T cells after infection in arsenic exposed mice [156]. On the contrary, Soto-Peña et al. found reduced TCD4+ T cell numbers and no cell count changes in CD8+ T cells

in exposed children [158]. Even though arsenic is believed to reduce the T cells' ability to fight against influenza infection, how arsenic specifically affects different T cell populations remains unknown given the inconsistencies across species.

Important immune responses against primary infection

CD8+ cytotoxic lymphocytes (CTLs) are one of the most effective immune cell types against viral infections, particularly during primary infection. CTLs can be activated and stimulated by APCs like DCs [163], as well as by their cytokines (e.g. IL-2) [164]. CTLs are essential for respiratory virus clearance [165]. Activated CTLs can recognize infected cells using epitopes of the viral proteins, and lyse the infected cells with perforin granzyme. Studies on CTL responses during IAV infections in mice have shown that the peak of viral-specific CTLs corresponds to the peak of viral clearance after infection [166]. On the other hand, CD4+ T cells were also identified to play multiple roles during viral infection [167]. Briefly, interferon-α/β from infected cells and IL-12 produced by APCs (including DCs and macrophages) stimulate the expression of T-box transcription factor 21 (T-bet) after viral infection [168-170]. T-bet then promotes the differentiation of Th1 subset from T cells [171]. One of the main functions of Th1 subset is to generate IFN-γ and TNF-α, which can induce cells, such as macrophages, to upregulate their phagocytic and antigen-presenting abilities [172]. Another important mechanism is to help CTLs respond to pathogens [173]. Therefore, environmental factors that target T cells may have a profound influence on immune responses against primary viral infection.

Arsenic exposure during pregnancy

Most of the aforementioned environmental factors are especially harmful to pregnant mothers because they could deteriorate the already unstable immune status of the mother [174-182].

Needless to say, some environmental compounds can pass through the placenta and adversely affect the immune system of the undeveloped fetus, due to their low competency to elicit immune responses [183]. When determining the correlations of arsenic exposure from drinking water between mothers, infants, and placentas in a human study, it was established that the placental arsenic concentration positively correlates with that of the mother [184]. A high concentration of arsenic is also found in the toenails and urine in both the mother and the infant [184]. This serves as evidence that the fetus could be exposed to arsenic through the mother. It is believed that Aquaporin 9 (AQP9) plays an important role in arsenic exposure in fetuses [185]. Studies have confirmed that AQP9 is an arsenic transporter [186, 187], and the expression level of AQP9 has been shown to enhance the effect of arsenic on cultured cells, including human placenta [188, 189]. Altogether, these discoveries suggest that AQP9 is related to arsenic uptake in fetuses. In a study on arsenic exposure during pregnancy in Bangladesh, researchers discovered that 70% of water wells that supply drinking water far exceeded the World Health Organization (WHO) arsenic concentration guideline of 10μg/L, and that low levels of arsenic exposure (<100μg/L) in urine were associated with reductions in birth weight, head circumference, and chest circumference [190]. Similar effects were found in mice as well. Kozul et al. confirmed that arsenic effects on the dams have a significant influence on body weight in the offspring [191]. *In utero* arsenic exposed offspring showed a significant decrease in weight as compared to offspring without arsenic exposure. Additionally, more irreversible effects can occur especially on the immune system in the developing fetus [192, 193]. For this reason, most studies focus on the health of the fetus while the consequences of arsenic

exposure on mothers have not equally been emphasized, even though it was found that mothers are also highly susceptible to arsenic exposure during pregnancy [194].

HYPOTHESIS & AIMS

The effects of arsenic exposure on a mothers' immune system during pregnancy are not well understood. It is known that pregnant mothers have attenuated immune functions [195], and arsenic exposure could deteriorate their immunity [196, 197]. However, it is unclear how the combination of pregnancy and arsenic exposure affects maternal health. The focus of my study is to investigate how arsenic affects the health of pregnant females in the context of a primary influenza A virus (IAV) infection. **My hypothesis** is that arsenic exposure during pregnancy will significantly increase the risk of infectious diseases and that the IAV disease pathology will be more severe than under any of the two conditions alone (arsenic exposure or pregnancy). I have tested this hypothesis by addressing the following three aims: **Aim 1.** Assess whether arsenic exposure during pregnancy increases the IAV load in the pregnant mice; **Aim 2.** Determine whether arsenic exacerbates the innate and adaptive cytokine/chemokine responses in response to IAV infection; **Aim 3.** Identify whether innate immune cells (macrophages and neutrophils) and adaptive immune cells (T cells, both CD4+ and CD8+ and B cells subpopulations) are altered in number by arsenic exposure in pregnant dams infected with IAV. The goal of this study is to determine whether there is a **synergistic effect of arsenic and pregnancy on IAV immune response outcomes.** A schematic diagram (Figure 2) for my research is included below.

MATERIALS & METHODS

Animal model:

Male and female C57BL/6NCrl mice aged 6-8 weeks were purchased from Charles River Laboratories (Frederick, MD) and were given 1 week to acclimate before experiments began. All mice were co-housed and did not exceed 5 animals per cage. Each cage was maintained in a temperature- and humidity-controlled facility with a fixed light-dark cycle (14-hour light and 10-hour dark). Food and water were provided *ad libitum*. A low arsenic chow diet was obtained from Research Diets (AIN-93M) and water was spiked with known concentrations of sodium(meta)arsenite (Millipore Sigma, St. Louis, MO). Male mice were weighed once a week. Female mice were weighed once a week before breeding, and every day after infection. Mice were euthanized if they were found to have lost more than 35% of their starting body weight before being challenged with IAV. This study was approved by the Animal Care and Use Committee to utilize this non-standard weight loss cut-off since studies are showing that mice can recover from such weight loss after influenza infection [198-200]. All animal procedures were conducted under the Johns Hopkins University Animal Care and Use Committee protocols M017H323.

Arsenic exposure

Mice were provided with 100ml of freshly-prepared water per cage twice weekly. Water was prepared using Crystal Springs (Lakeland, FL) purified, arsenic-free water. Concentrated arsenic water (10,000ppb of sodium(meta)arsenite) was used to make fresh 100ppb arsenic water. Both arsenic water and pure water were given to mice in graded water bottles, remaining food

and water were weighed or measured and recorded during water and food change to assess consumption across treatment groups. Cages were randomly assigned to receive 0 or 100ppb iAs through their drinking water daily for 3.5 weeks before and throughout infection.

Virus Growth, Purification, and Quantification

Mouse-adapted H1N1 influenza A/California/04/09 (ma2009) generated by Dr. Andrew Pekosz from a published sequence [201] was used for primary influenza infection. The virus was cultured in Madin-Darby canine kidney (MDCK) cells that were also a generous gift of Dr. Andrew Pekosz. MDCK cells were maintained at 37°C in Dulbecco's Modified Eagle's Medium (DMEM; Sigma-Aldrich, St. Louis, MO) supplemented with 1% L-glutamine and 10% fetal bovine serum (FBS; Sigma-Aldrich, St Louis, MO), unless otherwise stated.

To purify the virus, 90-100 percent confluent T150 flasks of MDCK cells were gently washed twice with phosphate-buffered saline (PBS; Quality Biological, Gaithersburg, MD), then inoculated with 5ml of virus-containing DMEM (supplemented with 1% L-glutamine, 0.5% BSA and 1µg/ml N-acetyl trypsin at a multiplicity of infection (MOI) of 0.1). Cells were then incubated with the viral media at room temperature for 1 hour on an orbital rocker table. After 1 hour, viral media in each flask was replaced with 18ml of DMEM supplemented as described earlier in this section. Cells were then incubated at 32°C for 4 days or until cells had sloughed off the bottom of the flasks, at which point the media was collected in 50ml conical tubes and centrifuged at 500 x g for 10 minutes at 4°C to remove the MDCK cell pellet. Viral supernatant could then be titered according to the 50% tissue culture infective dose ($TCID_{50}$) described below for further infections.

Anesthesia and Viral infection

Mice were anesthetized with a 100µl mixture of ketamine (80mg/kg) with xylazine (13mg/kg), and PBS via intraperitoneal (*ip*) administration and maintained on a small heating pad before viral infection. Mouse-adapted Influenza A/California/04/09 (ma2009 H1N1) was used for IAV infection [202]. The actual dose for infection was 10 $TCID_{50}$, which was diluted from a ma2009 H1N1 stock (10^7 $TCID_{50}$) in DMEM. When the mice were deemed insensate via toe pinching, they were inoculated intranasally with either 30µl of virus-containing DMEM or 30µl of plain DMEM as control. Mice were monitored until fully recovered. After infection, body weight, rectal temperatures of female mice, and their clinical scores were recorded daily until sacrifice. The calculation of clinical score was based on the cumulative score of the signs of piloerection, dyspnea, lack of escape response, and hunched poster. Each sign scored 1 point and the total score (out of a possible score of 4) represents the clinical score for each mouse. Mice were euthanized if they were found to have lost more than 35% of their starting body weight at the time of infection. All experiments were terminated at 8 days after infection.

Experimental design

Female C57BL/6NCrl mice were exposed to 100ppb of iAs in the form of sodium(meta)arsenite through drinking water *ad libitum* for 3.5 weeks prior to breeding and throughout gestation (Figure 3). Female mice were mated 1:1 with non-iAs exposed male C57BL/6NCrl mice for 1 night. Dams were infected with ma2009 H1N1 on gestation day 10 and sacrificed on day 18 of pregnancy for sample collection. Hence, the mice had a total of 5 weeks of arsenic exposure by the day they were infected with ma2009 H1N1. On the day of sample collection, BALF, lungs, and mediastinal lymph nodes (MLNs) were collected to perform further analysis. Since samples

were collected on gestation day 18, the maternal immune response during the early third trimester was assessed [203].

Bronchoalveolar lavage fluid (BALF) was collected for cytokine analysis

After the right lungs were tied off with thread, the left lungs were lavaged with 5ml of PBS. The collected lavage fluids were centrifuged at 500 × g for 6 minutes at 4°C, and the supernatants were stored at -80°C until analysis via the Luminex assay was performed. The cell pellets were resuspended with 100µl FBS-supplemented with PBS and transferred back into the rest of the digested lung samples for flow cytometry.

Left lungs were collected for immune cells analysis after lung digestion

Lavaged left lungs were transferred to C-tubes (Miltenyi, Bergisch Gladbach, Germany) with 1ml of digestion buffer (1ml DMEM, 0.025g collagenase, 0.005g DNase per sample and sterile filtered once mixed). C-tubes were placed on a gentleMACS (Miltenyi, Bergisch Gladbach, Germany) for 1 cycle of dissociation then incubated for 30 minutes at 37°C. C-tubes were placed back on gentleMACs for a second cycle of dissociation. The dissociated lungs were transferred to 50ml conical tubes in the biosafety cabinet and filtered using a 70µm filter. Sterile 5ml syringe plungers were used to mash any remaining pieces of the lung through the filter. Filters were then rinsed with 5ml of PBS complemented with 10% FBS. 50ml conical tubes were centrifuged at 500 x g for 6 minutes at 4°C. Supernatants were pipetted off and 1ml of red blood cell lysis buffer was added. After 5 minutes of incubation on ice, 10ml of PBS with 10% FBS was added to the tubes and centrifuged at 500 x g for 6 minutes at 4°C. Supernatants were discarded. The pellets were resuspended with 1ml of complete DMEM (1ml DMEM without phenol red, 10% FBS, 1% L-glutamine (Gibco, Grand Island, NY), 1% Penicillin/Streptomycin

(P/S) HEPES (Gibco, Grand Island, NY), 1% sodium pyruvate (Gibco, Grand Island, NY), 1% non-essential amino acids (Gibco, Grand Island, NY)). Resuspended pellets from BALF collection were added back into the total lung cell suspension. Total cell counts were determined using a hemocytometer after being stained with trypan blue (Gibco, Grand Island, NY).

Right lungs were collected for virus titration after lung homogenization

Unlavaged right lungs were transferred to 5ml FACS tubes with 0.5ml of serum-free DMEM. A homogenizer was used to emulsify the lungs for 5 seconds on high speed followed by 5 seconds on the ice, and an additional 5 seconds on high speed. The homogenates were centrifuged at 2500 rpm for 10 minutes at 4°C. The supernatants were aliquoted into 100µl per tube and kept at -80°C until $TCID_{50}$ assays were performed.

Mediastinal lymph nodes (MLNs) were collected for immune cells analysis

Harvested MLNs were placed in petri dishes with PBS and 10% FBS, and ground with frosted microscopy slides. Single-cell suspension from MLNs was centrifuged at 500 x g for 6 minutes at 4°C. Pellets were resuspended with 1ml of complete DMEM medium as previously described for the left lung protocol. Cell counts were determined using a hemocytometer after cells were stained with trypan blue. Supernatants were collected and adjusted for cell count for later use in flow cytometry analysis.

$TCID_{50}$ assay

$TCID_{50}$ (Median Tissue Culture Infectious Dose) is a method used to determine viral titers. This assay calculates the dilution of a virus that kills 50% of the total wells of cultured kidney cells [204, 205]. For optimal results, Madin-Darby canine kidney (MDCK) cells need to be 90-100%

confluent in the 96 well plates before being used. Each well was washed three times with 200µl of Calcium/Magnesium-supplemented PBS. Virus containing lung homogenate supernatants were diluted 10-fold and added in replicates of 6 with infection media (0.5% of 10% BSA and 5µl of NA Trypsin per 10ml infection media). Plates were incubated for 6 days at 32°C. Cells were fixed with Z-fix (10% aqueous buffered zinc formalin) and stained with naphthol blue black (Sigma-Aldrich, St. Louis, MO) overnight prior to being washed with distilled water. Wells with dead cells were identified and $TCID_{50}$ was calculated using the Reed-Muench method [199].

Multiplex cytokine/chemokine assay

BALF concentrations of 32 cytokines were measured using Milliplex magnetic bead kit MCYTOMAG-70k-32 (Millipore, Billerica, MA). A Luminex MAGPIX system (Bio-Rad, Hercules, CA) was used to analyze all samples in conjunction with a Curiox plate washer and DropArray™ microplates (Curiox, Woburn, MA). All assays were performed according to the appropriate Curiox protocol [206]. A comprehensive analysis provided insights on how immune responses were affected in the following 32 cytokines and chemokines assessed in the mice samples: Eotaxin, G-CSF, GM-CSF, INF-γ, IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-12(p40), IL-12(p70), IL-13, IL-15, IL-17, IP-10, KC, LIF, LIX, MCP-1, M-CSF, MIG, MIP-1α, MIP-1β, MIP-2, RANTES, TNFα, and VEGF. Briefly, a Drop Array-96 plate was incubated for 30 minutes with blocking buffer (PBS with 1% BSA) after which each well was loaded with BALF samples and magnetic beads, and the plate was incubated with shaking (350 rpm) overnight at 4°C. Next, the plate was washed 3 times with wash buffer (PBS with 0.1% BSA and 0.05% Tween-20). Detection antibodies were added to each well prior to another incubation with shaking for 1 hour at room temperature, followed by 30 minutes of incubation with streptavidin-

phycoerythrin shaking at room temperature on the shaker. Finally, the plate was read using a Magpix and analyzed through xPONENT® software (Luminex Corporation, Austin, TX). Plate readings were recorded as magnetic bead counts. Data below the limit of detection, or bead counts lower than 20, were excluded.

Flow cytometry

Collected cells were suspended at 2 x 10^6 cells/ml in DMEM supplemented with 10% FBS and 1% L-glutamine, 1% P/S HEPES, 1% sodium pyruvate, 1% non-essential amino acids. Cells were then plated in round-bottom 96-well plates (100µl/well). Next, stimulations were completed to determine the function of immune cells. Panels for general immune cells, macrophages, and epithelial/endothelial cells were stimulated *ex vivo* with lipopolysaccharide (LPS) and IFN-γ in phenol-free DMEM for 18 hours at 37°C; B Cell Panel and Germinal Center B (GCBC)/Tfh Cell Panel were stimulated *ex vivo* with LPS in phenol-free DMEM for 18 hours at 37°C; T helper Cell Subset Panel was stimulated *ex vivo* with staphylococcus enterotoxin B (SEB) or influenza-specific peptide (CD8: $NP_{366-374}$ and CD4: $NP_{311-325}$) in phenol-free DMEM for 1 hour at 37°C, followed by adding Brefeldin A (GolgiPlug; BD Biosciences, San Jose, CA) to each well at a 1:1000 dilution, and incubated for 5 hours at 37°C. All plates were washed with PBS and resuspended in 100µl/well of mixture of zombie aqua viability dye (Biolegend) and PBS (1:1000 dilution) to determine viability. All plates were incubated in the dark for 15 minutes at 4°C. Cells were then washed with PBS and resuspended in 50µl/well of mixture of Fc block and FBS-supplemented PBS (1:50 dilution). All plates were incubated in the dark for 15 minutes at 4°C. 50µl/well of staining mixtures (antibodies and FBS-supplemented PBS) was then added according to the panels and incubated in the dark for 30 minutes at 4°C. Based on the panels,

cells were then stained with the following antibodies. **T helper Cell Subset Panel**: anti-CD8+Tetramer-PE, anti-CD38-PE-CF594, anti-CD4-PerCP-Cy5.5, anti-CD44-PE-Cy7, anti-CD8-AF700, anti-CD3-APC/Fire750, anti-CD183-BV421, and anti-CD196-BV605; **B Cell Panel:** anti-CD20-FITC, anti-CD24-PE-CF594, anti-CD23-PerCP-Cy5.5, anti-CD19-PE-Cy7, anti-CD27-APC, anti-CD38-AF700, anti-CD3-APC/Fire750, anti-IgG-BV421, anti-CD21-BV605 and anti-CD86-BV786; **Germinal Center B/Tfh Cell Panel:** PNA-FITC, anti-CD185-PE, anti-CD11c-PE-CF594, anti-CD4-PerCP-Cy5.5, anti-CD19-PE-Cy7, anti-CD103-AF700, anti-IgG-BV421 and anti-PD1-BV786; **General Immune Cell Panel:** anti-CD64-FITC, anti-Siglec-F-PE, anti-CD11c-PE-CF594, anti-CD86-PerCP-Cy5.5, anti-CD19-PE-Cy7, anti-CD27-APC, anti-CD8-AF700, anti-CD3-APC/Fire750, anti-Ly-6C-BV421, anti-CD11b-BV605 and anti-F4/80-BV785; **Macrophage Panel:** anti-CD64-FITC, anti-Siglec-F-PE, anti-CD24-PE-CF594, anti-CD86-PerCP-Cy5.5, anti-CD19-PE-Cy7, anti-TLR2-APC, anti-CD11c-AF700, anti-I-A/I-E-APC/Fire750, anti-Ly6C-BV421, anti-CD11b-BV605 and anti-F4/80-BV785; **Epithelial/Endothelial Cell Panel:** anti-CD45-FITC, anti-CD54-PE, anti-CD326-PE-CF594, anti-CD86-PerCP-Cy5.5, anti-CD19-PE-Cy7, anti-CD34-AF647, anti-CD11c-AF700, anti-I-A/I-E-APC/Fire750 and anti-CD31-BV421). After incubation, cells were washed with PBS and resuspended in 100µl/well of BD FACS-lysing buffer. All plates were incubated in the dark for 10 minutes at 4°C. All plates were then sealed with plate sealant, covered with tinfoil and frozen at -80°C.

There was an additional staining step for the T helper Cell Subset Panel and Germinal Center B (GCBC)/Tfh Cell Panel to stain intracellular cytokines and transcription factors, and incubations were all completed in the dark. Briefly, plates were thawed in the dark at room temperature and washed with FBS-supplemented PBS. Plates for T helper Cell Subset Panel were

resuspended in 100µl/well of BD Cytofix/Cytoperm solution and incubated for 20 minutes at 4°C. Plates were then washed with 100µl/well of BD Perm/Wash buffer and resuspended in 50µl/well of a mixture of antibodies (anti-IFN-γ-FITC, anti-FoxP3-APC, and anti-TNFα-BV785) and BD Perm/Wash buffer. Plates were incubated for 30 minutes at 4°C. After incubation, plates were washed with 100µl/well of BD Perm/Wash buffer and resuspended in 100µl/well of FBS-supplemented PBS. Plates were stored at 4°C and ready for analysis on the same day or the next day. Plates for Germinal Center B (GCBC)/Tfh Cell Panel were resuspended in 100µl/well of Biolegend Fix/Perm solution and incubated for 20 minutes at room temperature. Plates were first washed with 100µl/well of FBS-supplemented PBS and then 100µl/well of Biolegend Perm buffer. Plates were resuspended in 100µl/well of Biolegend Perm buffer and incubated for 15 minutes at room temperature. Plates were centrifuged and resuspended in 100µl/well of a mixture of antibody (anti-Bcl-6-AF647) and Biolegend Perm buffer. Plates were then incubated for 30 minutes at room temperature. After incubation, plates were washed and resuspended with 100µl/well of FBS-supplemented PBS. Plates were stored in the dark at 4°C and ready for analysis on the same day or the following day.

Cell data was acquired through Fortessa fluorescence-activated cell sorter with FACSDiva software (BD Biosciences, San Jose, CA), and analyzed with FlowJo software (Flowjo, Ashland, OR). The number of target cells was calculated by multiplying total cell counts by the percentage of the live cells in live-cell gating and multiplied by the percentage of the target cell among the live cell population.

Statistical Analysis

Viral titers, cytokine concentrations, and flow cytometric analyses were analyzed by two-tailed

unpaired Student's t-test (t-test) with a 95% confidence interval. Statistical significance was considered at $P<0.05$. Statistical analysis was performed using Prism 8 (Graphpad Software Inc, La Jolla, CA).

RESULTS

Chronic iAs exposure has no effect on influenza A virus titers in pregnant mice

In order to test the hypothesis that chronic iAs exposure weakens immune responses against IAV infection in pregnant mothers, viral titers of lung homogenates were determined by $TCID_{50}$ assays in arsenic exposed and control pregnant mice at 8 days post IAV infection. No significant viral titer differences were observed between the two groups (Figure 4). However, although not significant, unexposed pregnant mice showed increased viral titers as compared to non-pregnant female mice after IAV infection (*p* value = 0.3487).

Chronic iAs exposure did not alter levels of cytokines or chemokines in pregnant mice with IAV infection

To verify whether chronic iAs exposure would alter the expression of certain cytokines during immune responses against IAV infections in pregnant mothers, cytokine concentrations were assessed in BALF specimens using a multiplex cytokine/chemokine assay. No significant cytokine level difference was observed among the 32 targets between the arsenic-exposed and unexposed pregnant group 8 days after infection (Figure 5). Pro-inflammatory cytokines (e.g., TNF-α (*p* value = 0.5888)) and anti-inflammatory cytokines (e.g., IL-6 (*p* value = 0.9574) and IL-10 (*p* value = 0.3416)) showed no sign of alteration by arsenic exposure in infected pregnant

mice compared to the controls. (Data for cytokines other than TNF-α, IL-6, and IL-10 are not shown.)

Chronic iAs exposure did not result in significant changes in immune cell counts (except AMs in MLNs) or activation in pregnant mice with IAV infection

To determine whether chronic iAs exposure would affect certain immune cell counts during immune responses against IAV infections in pregnant mothers, immune cell counts of lung and MLN homogenates were calculated through flow cytometry analysis. Six panels for different types of immune cells were evaluated. Alveolar macrophages in MLN homogenates were found to have a significant decrease in cell count in arsenic-exposed pregnant mice, as compared to unexposed pregnant mice 8 days after infection (*p* value = 0.0421). No statistically significant cell count or function (expression of activation markers or cytokines) difference was observed in the rest of the targeted immune cells between the arsenic-exposed and unexposed pregnant group 8 days after infection (Figure 6) and (Figure 7). For neutrophils, alveolar macrophages, dendritic cells, plasmacytoid dendritic cells, and cytotoxic T lymphocytes, two separate cell count results were presented for each cell type, which were based on the analyzed data from either MLN homogenates or lung homogenates. During influenza infections, cytotoxic T lymphocyte response against viral proteins are generated by dendritic cells that migrated from the lung to the MLN [207, 208], whereas neutrophils and alveolar macrophages were also found to recruit to lymph nodes to regulate immune responses when encountered pulmonary infections [209, 210]. Thus, how these cell types were affected in MLNs and lungs are important. Cell counts for B cells, germinal center B cells (GCBC), and T follicular helper cells (Tfh) were based on the data from MLN homogenates. This is because these cells respond to

influenza infections mostly in MLNs [211]; whereas cell counts for T helper cells, IAV+ cytotoxic T lymphocytes, and T regulatory cells were based on the data from lung homogenates, and this is because these cells are important proinflammatory effectors and regulators of inflammations in IAV-infected lungs.[212]

B cells are essential for humoral immunity [211] and can help fight influenza infection by neutralizing the virus directly or lysing infected cells. Despite the fact that B cells are not designed to immediately respond when pathogens are present, in mice, these cells can secrete antibodies a few days after being activated by antigens [213-215]. B cells that undergo maturation in the germinal center differentiate into plasma B cells and memory B cells [216]. B cells and memory B cells secrete antibodies and are important for rapid responses when encountered with the same infection again, respectively. However, cell counts of either total B cells (CD4-CD19+) (*p* value = 0.5429) or germinal center B cells (GCBC) (CD4-CD19+PNA+) (*p* value = 0.7595) did not display a significant difference between arsenic-exposed pregnant group and unexposed pregnant group 8 days after infection.

T follicular helper (Tfh) cells (CD4+CD19+PD1+CD185+) are important for the formation of the germinal center and the production of high-affinity antibodies and memory B cells [217]. These events can determine how effectively B cells respond to infections. However, a significant difference in cell count was only found between unexposed pregnant mice and unexposed non-pregnant mice (*p* value = 0.0155). Although not significant, there was also an inverse relationship between arsenic exposures and lower cell counts of Tfh cells and increased expression in the arsenic exposed pregnant group 8 days after infection (*p* value = 0.0940).

Interestingly, an increasing trend of Bcl6 expression was found in the exposed and infected pregnant mice as well (*p* value = 0.0650).

Neutrophils (CD19-CD3-NKp46-) are the first responders to sites of infection, and it has been reported that neutrophils are the main infiltrating cell population during immune response against IAV infection [218]. This protective role against IAV infection is also observed in mice [219, 220]. However, no significant cell count difference was found between the arsenic-exposed pregnant group and unexposed pregnant group 8 days after infection (in MLNs: *p* value = 0.1007, in lungs: *p* value = 0.6647).

Dendritic cells (DC) (CD19-CD3-NKp46-CD64-CD11C+CD11b+Ly6C+CD11b+) are important antigen-presenting cells for antigen-specific immune responses, and plasmacytoid dendritic cells (pDC) (CD19-CD3-NKp46-CD64-CD11C+CD11b-Ly6C+) are one of the subsets that is responsible for production of Type 1 IFNs after viral infections [221]. However, no significant differences were observed in either total DC (in MLNs: *p* value = 0.1928; in lungs: *p* value = 0.2456) or pDC (in MLNs: *p* value = 0.1469; in lungs: *p* value = 0.8831) cell counts between the arsenic-exposed and unexposed pregnant group 8 days after infection.

Macrophages are important innate immune cells against pathogens, and alveolar macrophages (AMs) (in MLNs: CD19-CD3-NKp46-CD64+CD11c+, in lungs: CD19-Ly6C-SiglecF+) in particular are critical in controlling the replication and the spread of viral infection [222]. A significant decrease in cell count was found in AM populations between the arsenic-exposed and the unexposed pregnant group 8 days after infection in MLNs (*p* value = 0.0421). However, no significant cell count difference was found in the lungs (*p* value = 0.8220). Additionally,

expression of activation marker after the stimulation (LPS and IFN-γ) didn't change in infected pregnant mice with exposure (in lungs: *p* value = 0.8599). Though not statistically significant, there was a decreasing trend in which infected pregnant mice in both arsenic exposed (in lungs: *p* value = 0.2244) and unexposed group (in lungs: *p* value = 0.3905) showed a reduced number of AMs, as compared to infected non-pregnant mice.

During viral infection, T helper cells (CD3+CD4+) are essential for antibody production by B cells and CD8+ cytotoxic T lymphocytes (CTLs) activation [223, 224]; whereas CTLs (in MLNs: CD19-CD3+CD8+CD11c-, in lungs: CD3+CD8+) are important for viral clearance [225]. Once viral antigen is acquired, IAV-specific CTLs (IAV+ CTLs) (CD3+CD8+ma2009+) are responsible for killing viral-infected cells [146]. On the other hand, T regulatory (CD3+CD4+Foxp3+) cells are important suppressive T cell subsets that attenuate CD4+ T helper cells and CTLs proliferation and cytokine production [226]. However, no significant cell count difference was observed in T helper cells (*p* value = 0.4981), CTLs (in MLNs: *p-value*=0.3986, in lungs: *p* value = 0.5212), T helper cells: CTLs ratio (in lungs: *p* value = 0.1092), IAV+ CTLs (*p* value = 0.1092), and T regulatory cells (*p* value = 0.5449) between the arsenic-exposed and unexposed pregnant group 8 days after infection.

DISCUSSION

Our study tested the hypothesis that arsenic exposure during pregnancy would significantly increase the risk of infectious diseases and that the disease pathology would be more severe than under any of the two conditions alone (arsenic exposure or pregnancy). Other than the

significant cell count decrease of the AMs in MLNs (Figure 6), no significant differences were found in viral titer (Figure 4), cytokine responses (Figure 5), nor immune cell counts and function in the rest of the targeted immune cells (Figures 6 and 7).

There are currently no studies on how AMs are affected by chronic arsenic exposure in IAV infected pregnant mice. However, it's has been reported that AMs are crucial for regulating the disease severity of IAV infections and the development of lethal pulmonary injury [227-230]. AMs are located at the terminal airways, which makes them an important first-line defense against many lower respiratory infections, including IAV infections [231]. Based on a study on AMs, Schneider et al. suggested that the main function of AMs is to remove dead cells and cellular debris, which can avoid blocking the airways and maintain gas exchange during IAV infections [232]. Additionally, they also suggested that AMs may be associated with viral clearance through unknown mechanisms. According to Kirby et al., AMs were also discovered to migrate from the lungs to the MLNs after bacterial infections, and they suggested that AMs may be responsible for the earliest delivery of pathogens to secondary lymphoid tissue instead of DCs. [233]. Our findings suggest that arsenic exposures may affect the immune responses against IAV infections in pregnant mice by reducing the number of AMs in MLNs (Figure 6). However, we found no significant difference in subsequent cytotoxic T cell proliferation (Figure 6) or activation (Figure 7) between arsenic-exposed and unexposed pregnant females.

Surprisingly, there was no viral titer difference between arsenic exposed and unexposed pregnant mice. It is not known why arsenic exposure did not induce higher viral titer in pregnant mice, but this is not due to the infection stage being compromised. Undetectable virus titer in the uninfected groups and a significant increase in infected groups indicate that

the IAV infection was performed successfully. The increased viral titer in the infected unexposed pregnant mice compared to non-pregnant mice, maybe due to immune modulation during pregnancy. According to a study on adverse pregnancy outcomes from the H1N1 influenza virus, pregnant BALB/c mice with mouse-adapted A/Brisbane/59/07 (H1N1) virus infection had a higher viral load in the lungs than non-pregnant BALB/c mice with the same viral infection [234]. It is suggested that a special endocrine environment that maintains pregnancy reduces immune responses against infection. However, the assumption that pregnancy attenuates immune responses against infection and results in viral clearance deficiency may not be as simple. According to Vermillion et al., factors that may lead to an absence of viral differences between infected pregnant mice and non-pregnant mice include variations in virus replication efficiency, inflammatory cell recruitment, and pulmonary cell repair [235].

This study also elucidated that progesterone can induce pulmonary epithelial cell repair [236]. Since the integrity of epithelial cells is crucial for the innate immune response [237], the significant increase of progesterone in pregnant mice may be one essential factor for keeping up the immunity against IAV infection in non-pregnant mice. Another potential explanation for why we didn't observe any significant changes in immune response upon arsenic exposure is related to the timing of sample collection. In humans, it is widely accepted that pregnancy trimesters have different effects on the mother's immune response [238]. Generally, the second trimester is a stage where anti-inflammatory responses dominate over pro-inflammatory responses, whereas the inverse is observed during the third trimester [238]. Even if the infected pregnant mice have higher viral titer during the second trimester, the viral clearance capacity may have been restored by the time of tissue collection [239]. Because the

mice were challenged on gestation day 10 (during the second trimester) and tissues were collected on gestation day 18 (during the third trimester), the anti-inflammatory effects on immune responses against IAV infection may not have been ideal during this timeframe.

According to Kozul et al. study, viral titer differences between arsenic-exposed mice and non-exposed mice is significant at day 7 post-infection [156]. One of the possible reasons for not achieving a significant difference between infected control and arsenic exposed female mice in this study may be due to the relatively low viral titer of the virus used for infection. Kozul et al. used one half of the lethal dose for viral infection [156], which is about 45 $TCID_{50}$ [240, 241], whereas this study employed a 10 $TCID_{50}$ for viral infection. The dose used in our study to infect the mice may be too low for arsenic to exhibit its impact on immune responses against viral infection.

When influenza virus infection occurs, the host's immune response goal is to contain the spread and eventually eradicate the virus [242]. Therefore, viral load can be a good indicator for comparing how arsenic exposure alters a pregnant mother's immunity against IAV infection. Since a viral titer difference was not observed between arsenic exposed and unexposed pregnant mice after infection, this may explain why targeted cytokines and immune cells (except AMs) also did not exhibit any significant concentration and cell count difference. Immune cells and cytokines together are responsible for proper immune responses against IAV infections [243]. It is common to observe changes in immune cell counts and cytokine concentration when studying immune effects from arsenic exposure and pregnancy [244]. Innate immune cells including neutrophils, dendritic cells, and macrophages are crucial during influenza infections by serving as the first line of defense against evading pathogens and

activating adaptive immune responses [218, 221, 222]. Adaptive immune cells, including B and T cell subsets, are pathogen-specific immune cells that are responsible for efficient responses against influenza infections [146, 211, 216, 217, 223-226].

Pro-inflammatory cytokines, including TNFα, are important components for anti-influenza responses, and TNFα has been recognized for its greater anti-influenza virus activity than gamma or alpha interferon [245]. On the other hand, anti-inflammatory cytokines, including IL-6 and IL-10, are important mediators that prevent excessive virus-induced inflammation during influenza infection [246, 247]. Without it, prolonged inflammation can lead to severe tissue damage or even death. IL-6 was found to protect neutrophils from virus-induced death [247], whereas TNFα was identified as a negative regulator in both innate and adaptive immune cells during viral infection [246]. Other than low infection dose, it's also possible that no significant difference in cytokine concentrations and immune cell counts between the arsenic exposed and control group was due to unknown mechanisms that interact with iAs during pregnancy.

Interestingly, there was a significant higher Tfh cell count in pregnant mice as compared to the non-pregnant mice (both unexposed and infected) (Figure 6). This is consistent with the findings in the Monteiro et al. study, which explained that pregnancy favors antibody production [248]. There was no significant difference between unexposed infected pregnant females versus arsenic-exposed infected pregnant females. However, the difference between non-pregnant females and pregnant females was ablated upon arsenic exposure (Figure 6). Future studies would have to investigate whether this would have any significant impact on humoral immunity during pregnancy.

LIMITATIONS & FUTURE DIRECTIONS

There are fundamental differences between animal models and humans which makes extrapolation of findings a huge barrier. For instance, mice have shorter pregnancies than humans. The estimated gestation time in mice is 19.5 days, whereas human pregnancies average 268 days (38 weeks and 2 days) from ovulation to birth [249, 250]. This directly results in different iAs exposure time during pregnancy. Additionally, the effect of chronic exposure on pregnancy may also be more severe in humans than mice as a result of iAs metabolism, which is more efficient and effective in mice than humans. Vahter et al. reported that humans excreted more MMA than any other metabolite in the urine, which indicates poorer iAs metabolism since a lower fraction of DMA in urine indicates higher retention in tissues [251]. On the other hand, mice can methylate arsenic efficiently and excrete up to 90% of the DMA in the urine in two days [252]. This significant difference in iAs metabolism rate may result in different iAs effects even when treated with the same dose.

The IAV dose (10 $TCID_{50}$) that was used for infection may not represent real-world exposures. However, it is difficult to accurately determine the dose for IAV infections that occurs in humans for various reasons. Factors including age, weight, and immunocompromised status can all affect susceptibility to IAV infection. O'Brien et al. found that mice infected with 10^5 $TCID_{50}$ of A/California/04/09 H1N1 virus resulted in up to 25% weight loss at 8 days p.i. [253]. In another study focused on the H1N1 virus's potential on neurotrophic and inflammatory effects in mice, researchers found significant weight loss occurred 8 days p.i. with a 10^3 $TCID_{50}$ non-mouse adapted A/California/04/09 H1N1 virus [254]. Weight loss is one important indicator of

clinical infection [255]. It may be worthwhile to not only use a higher virus dose but also utilize multiple viral doses to study IAV infection. By doing so, the significance between the treatment (iAs exposure) and the control group may be more apparent, and health outcomes from multiple viral doses can be more representative of the range of conditions experienced in real life. It is unlikely that every viral infection occurs with a similar amount of virus, so this hypothesis warrants further investigation.

In this study, mice were infected in the second trimester and sacrificed in the early third trimester. Despite maternal immune status switching back to proinflammatory dominant responses, the pregnant mice were predominantly under anti-inflammatory responses after infection. Since maternal immune status during pregnancy changes throughout pregnancy [38], different infection and sample collection time points may result in altered immune responses against influenza infection. If the duration between infection day and sample collection day remains the same, changing the infection day to either first or third trimester may possibly lead to different results. Generally, the immune status in the first and third trimester is believed to be shifted more towards Th1 responses than in the second trimester [238]. Hence, all the viruses may be eradicated by the time the sample is collected as the ability of viral clearance is enhanced by increased Th1 responses. However, more studies are needed to investigate altered immune responses during pregnancy, and how arsenic exposure might impact each trimester differently.

Since the $TCID_{50}$ assay did not establish viral titer differences between arsenic-exposed and unexposed pregnant mice, another way to measure IAV titer in each group is by quantifying the amount of IAV RNA through RT-qPCR (quantitative reverse transcription PCR) in the lung

homogenate [256]. Since the viral RNA indicates the amount of IAV that is present in the cell, and the only difference between the two groups is arsenic exposure, the group that possesses a higher amount of viral RNA is indicative of malfunctioning immune responses thus leading to low viral clearance. This speculation is in agreement with a previous study that characterized the correlation between viral load and influenza disease severity [257] and revealed that arsenic exposure increases the burden on the immune system because the influenza virus will no longer be properly contained by the immune system.

The Luminex assay is the most widely used assay to analyze the activity of numerous chemokines and cytokines [258]. Its biggest advantage is measuring multiple cytokines and chemokines simultaneously, which reduces not only the total time for processing and analysis but also the technical errors that may occur when analyzing multiple individual cytokines and chemokines. However, one potential drawback of the Luminex assay is that it focuses on the protein level of the target substance. If arsenic is affecting the cytokines and chemokines during the transcription or translation stage [259], the Luminex assay will not be able to capture these differences as it could only analyze the existing protein substances at a given time point. Therefore, if arsenic exposure induced a significant difference between arsenic-exposed and unexposed pregnant mice at the RNA level, RT-qPCR analysis on the cytokines and chemokines could help identify these changes. By combining the Luminex assay with RT-qPCR, more detailed arsenic effects that occur during the transcription and translation processes can provide valuable insights on how arsenic is affecting immune responses.

Overall, mice are sufficient to reflect what happens during human pregnancy [260] and have a similar compromised effect on the immune system from arsenic exposure [261]. However, gaps

still exist between these two species. To overcome this limitation, comparing the viral load of human samples can be useful to identify arsenic effects on the mother's immune responses against IAV infection during pregnancy in humans. This can be done by conducting real-time PCR on collected throat swab samples from IAV infected pregnant mothers with and without arsenic exposure [262]. If arsenic compromises immune responses against IAV infection, arsenic-exposed pregnant mothers with IAV infection should have a higher amount of virus than unexposed pregnant mothers with IAV infection [263].

Finally, a major limitation of this study is the limited numbers of animals that were available for certain treatment groups at the time of writing of this report, due to COVID-19 related lab closures. Therefore, these studies will have to be repeated to increase the sample number and subsequently the power of detection.

PUBLIC HEALTH RELEVANCE & CONCLUSION

Arsenic exposure produces a variety of devastating, and at times irreversible, health outcomes and diseases. Depending on the duration and the dose of exposure, arsenic exposures are generally divided into two types: acute high-dose exposure and chronic low-dose exposure [264]. However, diseases resulting from chronic low-dose exposure are much more complex [265-270]. With advanced surveillance technology, the likelihood of a significant amount of arsenic exposure is relatively low as compared to a low dose exposure scenario. Although places with high concentrations of arsenic still exist, most are closely monitored by authorities to effectively control and prevent hazards [271]. On the contrary, people are usually unaware

of chronic low-dose exposures [272]; yet people with chronic low-dose arsenic exposures are at high risk of developing diseases such as peripheral vascular diseases [273]. Unless the source of arsenic exposure is identified and mitigated, it is usually difficult to prevent the progression of such diseases. Pregnant women, in particular, are at high risk of being afflicted with infectious diseases [274] leading to severe outcomes or even death, if not treated properly. In 2009, the H1N1 influenza pandemic in the U.S. caused more infections in pregnant women than the general public [275]. The hospitalization rate was almost 5 times as much for pregnant women at a rate of 0.32 per 100,000 pregnant women, whereas it was 0.076 per 100,000 persons for the general public. In the first month of the outbreak, there were 6 deaths among pregnant women, accounting for 13% of total deaths in that group. This indicates that the influence of pregnancy on the immune system is substantial. Any additional interference on the pregnant mother's immune system can endanger their well-being critically

This study was aimed at assessing the severity of arsenic exposure on pregnant mothers. This work should inform the public about the consequences of arsenic exposure, especially the health problems that occur in mothers during pregnancy. Currently, most of the studies on health outcomes due to arsenic exposure during pregnancy have focused on the fetus, which is important because arsenic can affect the long-term health of the offspring. However, the health of the mothers should also be highly valued because pregnancy affects them greatly. The additional influence from arsenic exposure may place mothers in a worse predicament. Impacts of arsenic exposures on human health, including adverse reproductive outcomes and neurological disorders, are hard to recover from because of their irreversible characteristics [276]. When these incidents occur in pregnant mothers, these diseases can be worse due to the

delicate condition they are experiencing. To complete pregnancy, one of the requirements that pregnant mothers must fulfill is to reduce immune responses against allographs. This makes them more vulnerable to pathogens like influenza A virus. Despite this study showing no additional impact on IAV infection severity on pregnant mothers after chronic arsenic exposure, there is existing literature that provides evidence that arsenic interrupts proper immune responses against viral infection [277]. Thus, more research on arsenic effects on the immune responses against IAV infections in pregnant mothers is needed.

FIGURES

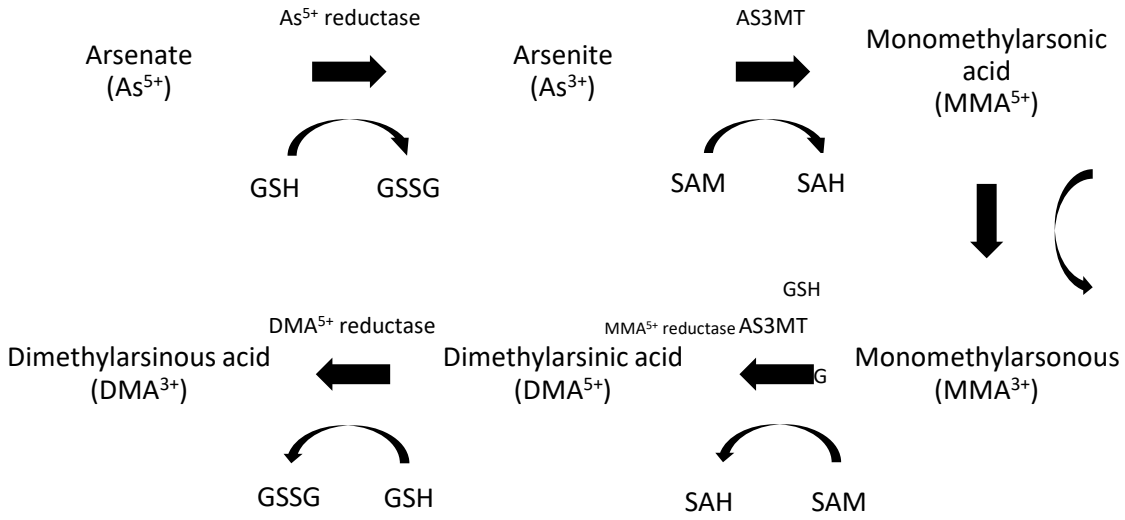


Figure 1. Inorganic arsenic metabolic pathway. GSH: glutathione, GSSG: Glutathione disulfide, SAM: S-adenosylmethionine, AS3MT: arsenic (+3 oxidation state) methyltransferase, SAH: S-adenosylhomocysteine. Information used to develop this figure comes from previous literature [112-114].

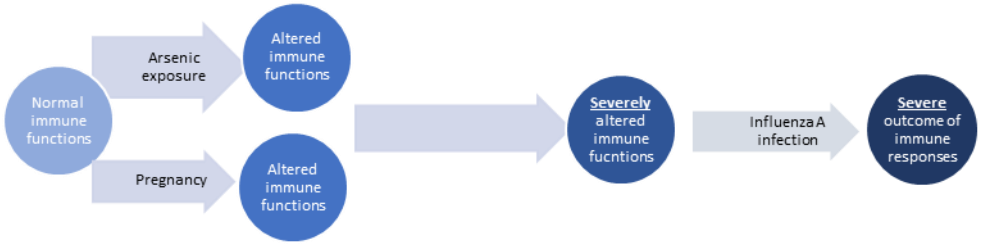


Figure 2. Schematic diagram of the anticipated effects of arsenic exposure in pregnant females during influenza A virus infection.

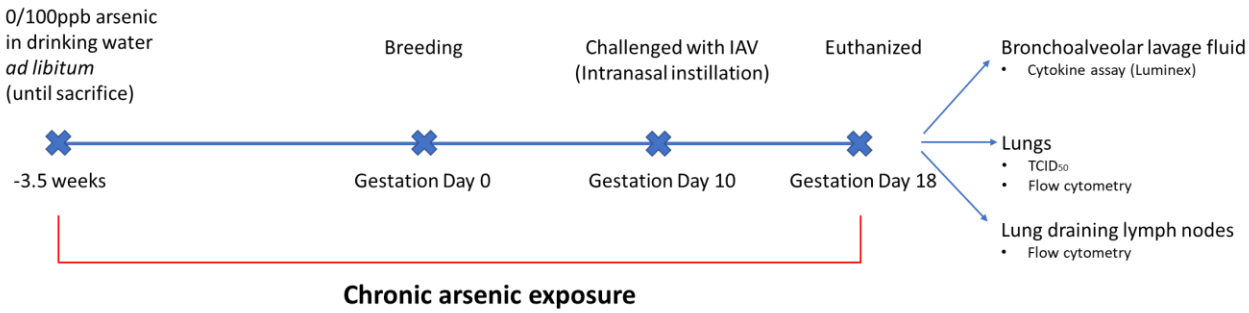


Figure 3. Timeline for arsenic exposure, breeding, infection, and sample collection. For the exposed group, female C57BL/6NCrl mice were exposed to 100ppb of iAs in the form of sodium(meta)arsenite through drinking water ad libitum for 3.5 weeks prior to breeding and throughout gestation; whereas female mice in the unexposed group were given plain water throughout the same course. Female mice were mated 1:1 with non-iAs exposed male mice for 1 night. Dams were infected with ma2009 H1N1 on gestation day 10 and euthanized on day 18 of pregnancy for sample collection. Bronchoalveolar lavage fluid was collected for cytokine assay; lungs were collected for Median Tissue Culture Infectious Dose ($TCID_{50}$) assay and flow cytometry; lung draining lymph nodes were collected for flow cytometry as well. IAV: mouse-adapted H1N1 influenza A/California/04/09 (ma2009).

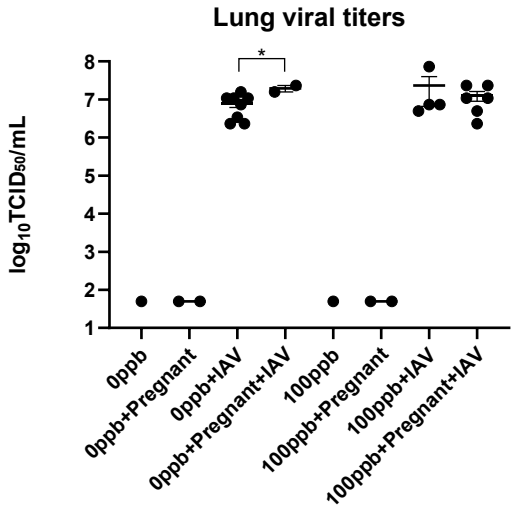


Figure 4. Viral titers of lung homogenates.
Homogenized left lungs were assessed for viral titers via $TCID_{50}$. Each dot represents data from one animal. 26 mice in eight different treatment groups. No treatment, n=1. Pregnant, n=2. IAV infected, n=8. Pregnant and IAV infected, n=2. 100ppb of arsenic, n=1. 100ppb of arsenic and pregnant, n=2. 100ppb of arsenic and IAV infected, n=4. 100ppb of arsenic, pregnant, and IAV infected, n=6. Samples were collected 8 days after infection (gestation day 18). * *p* value <0.05. IAV: mouse-adapted H1N1 influenza A/California/04/09 (ma2009).

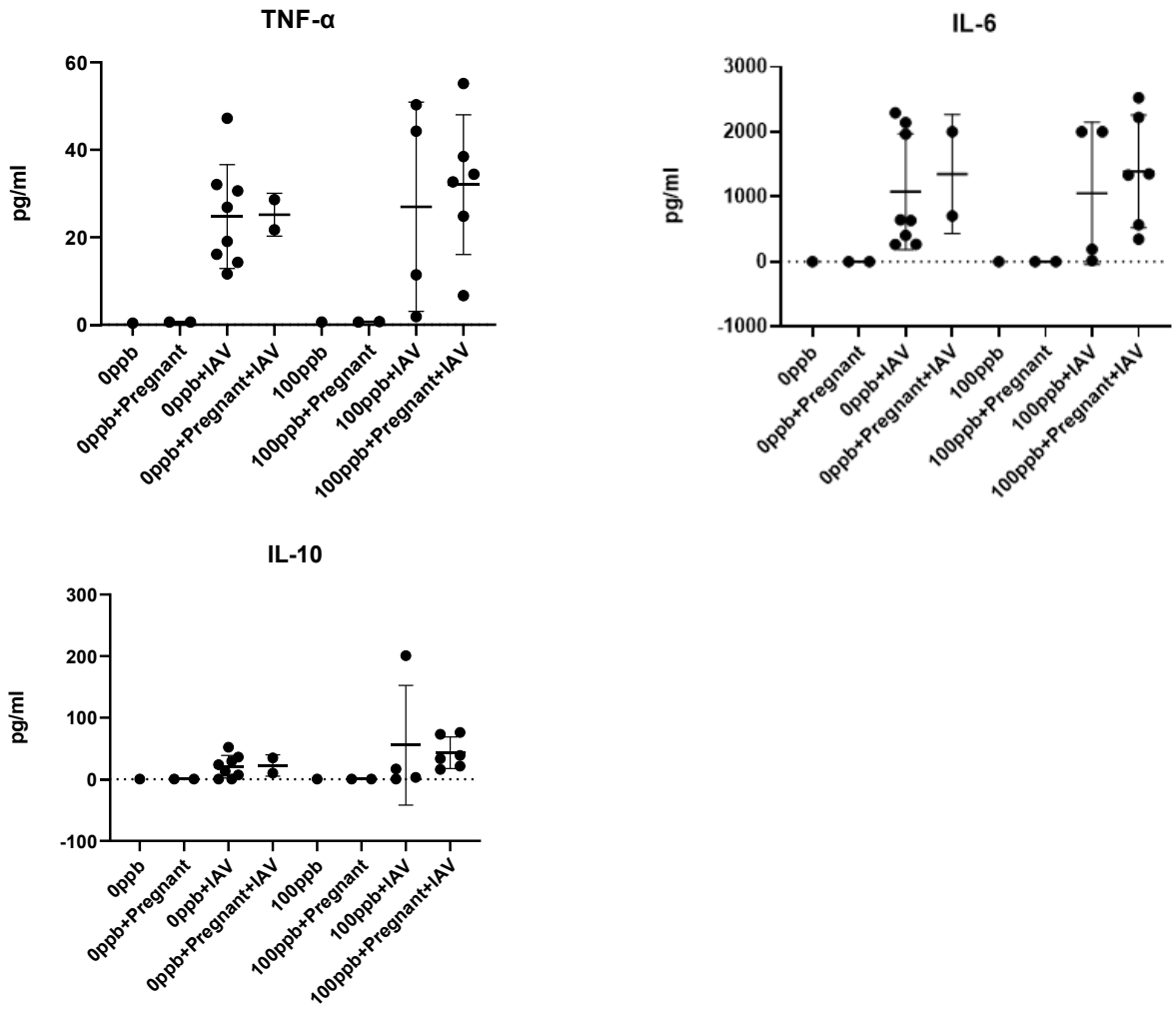
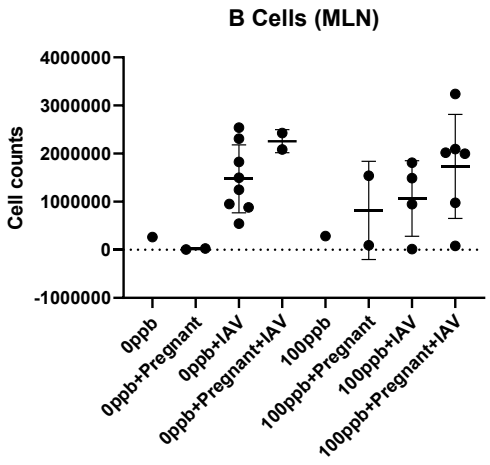
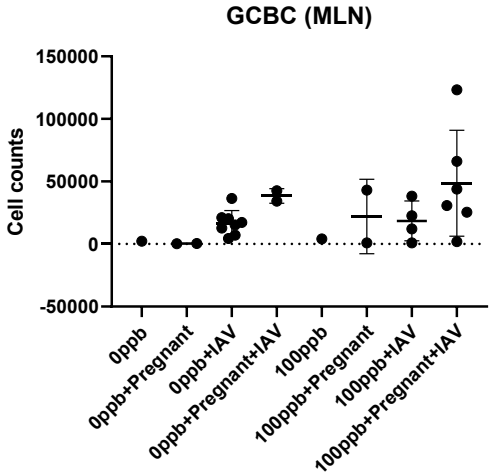


Figure 5. Cytokine concentrations in bronchoalveolar lavage fluid (BALF).
BALF was assessed for cytokine concentrations via a 32-multiplex Luminex assay. Tumor necrosis factor alpha (TNFα), IL-6, and IL-10 are important immune regulators during IAV-infections. TNFα (proinflammatory cytokine), as well as IL-6 and IL-10 (anti-inflammatory cytokines), are key cytokines that mediate inflammations during IAV-infections. No significant difference in concentrations in TNFα, IL-6, IL-10, and the other 29 cytokines/chemokines (data not shown). Each dot represents data from one animal. 26 mice in eight different treatment groups. No treatment, n=1. Pregnant, n=2. IAV infected, n=8. Pregnant and IAV infected, n=2. 100ppb of arsenic, n=1. 100ppb of arsenic and pregnant, n=2. 100ppb of arsenic and IAV infected, n=4. 100ppb of arsenic, pregnant, and IAV infected, n=6. Samples were collected 8 days after infection (gestation day 18). IAV: mouse-adapted H1N1 influenza A/California/04/09 (ma2009).


B Cells (MLN)
Cell counts
4000000
3000000
2000000
1000000
0
-1000000
0ppb
0ppb+Pregnant
0ppb+IAV
0ppb+Pregnant+IAV
100ppb
100ppb+Pregnant
100ppb+IAV
100ppb+Pregnant+IAV


GCBC (MLN)
Cell counts
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100000
50000
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-50000
0ppb
0ppb+Pregnant
0ppb+IAV
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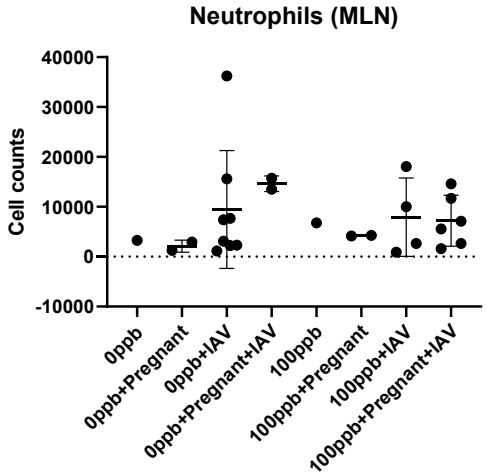
100ppb+IAV
100ppb+Pregnant+IAV


Neutrophils (MLN)
Cell counts
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30000
20000
10000
0
-10000
0ppb
0ppb+Pregnant
0ppb+IAV
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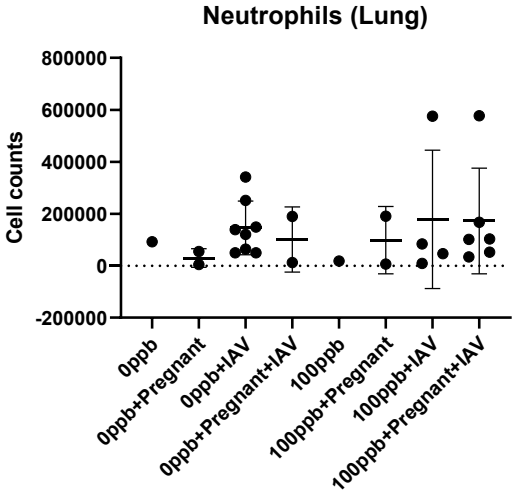
100ppb+IAV
100ppb+Pregnant+IAV


Neutrophils (Lung)
Cell counts
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600000
400000
200000
0
-200000
0ppb
0ppb+Pregnant
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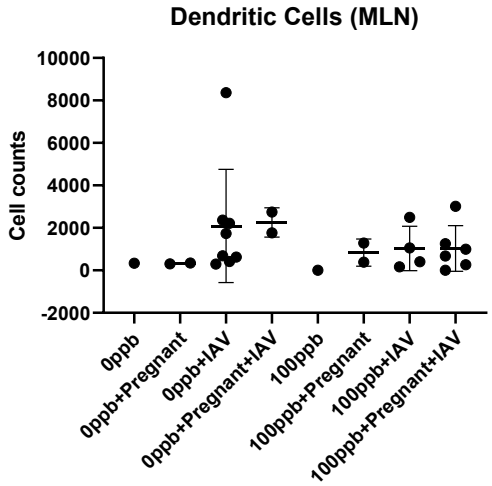
100ppb+IAV
100ppb+Pregnant+IAV


Dendritic Cells (MLN)
Cell counts
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8000
6000
4000
2000
0
-2000
0ppb
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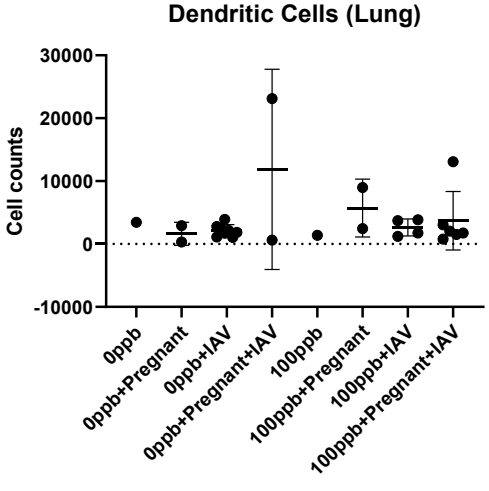
100ppb+IAV
100ppb+Pregnant+IAV


Dendritic Cells (Lung)
Cell counts
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20000
10000
0
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0ppb
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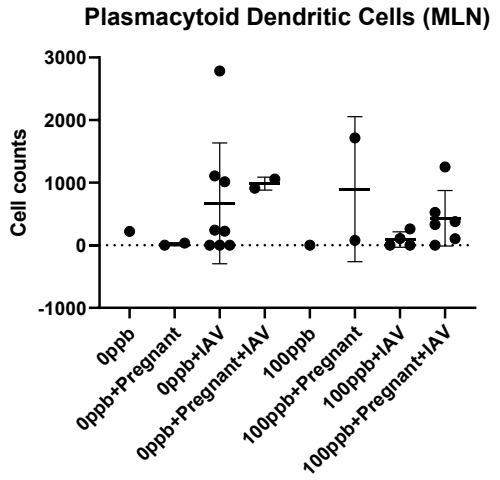
100ppb+Pregnant+IAV


Plasmacytoid Dendritic Cells (MLN)
Cell counts
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2000
1000
0
-1000
0ppb
0ppb+Pregnant
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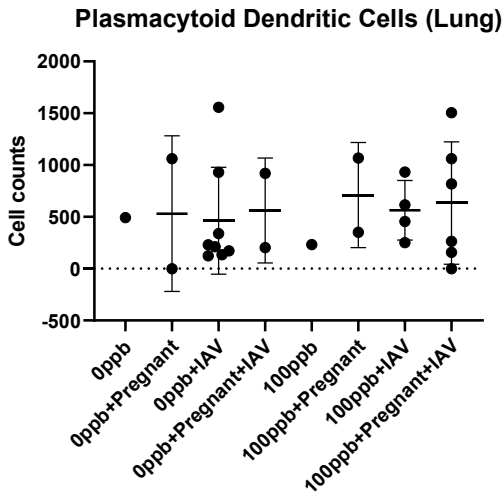
100ppb+IAV
100ppb+Pregnant+IAV


Plasmacytoid Dendritic Cells (Lung)
Cell counts
2000
1500
1000
500
0
-500
0ppb
0ppb+Pregnant
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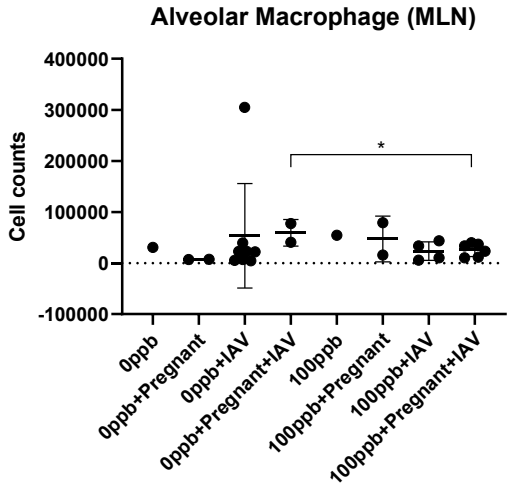
100ppb+IAV
100ppb+Pregnant+IAV


Alveolar Macrophage (MLN)
Cell counts
400000
300000
200000
100000
0
-100000
*
0ppb
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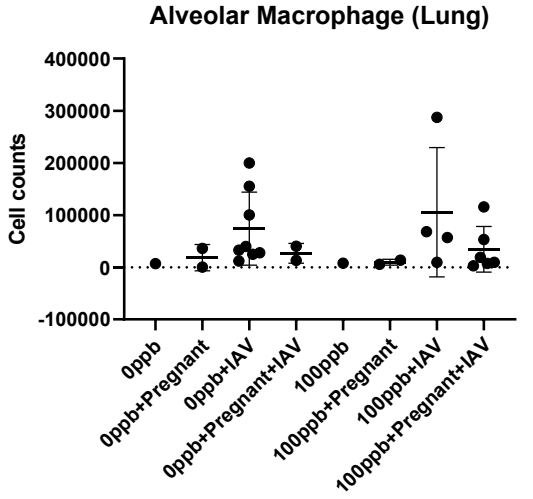
Alveolar Macrophage (Lung)
Cell counts
400000
300000
200000
100000
0
-100000
0ppb
0ppb+Pregnant
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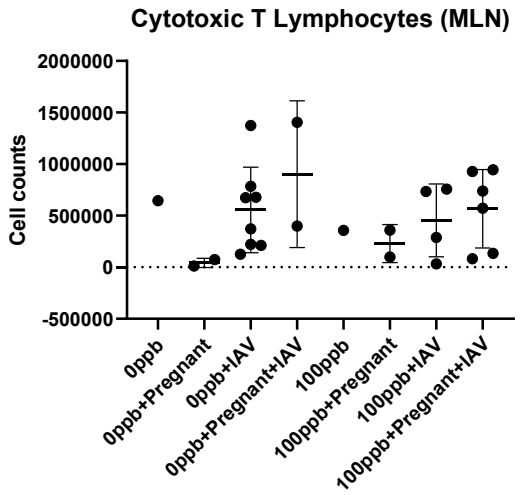
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Cell counts
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1000000
500000
0
-500000
0ppb
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100ppb
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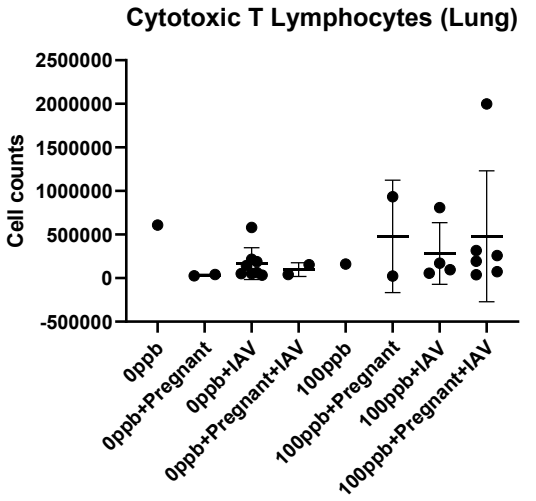
100ppb+IAV
100ppb+Pregnant+IAV


Cytotoxic T Lymphocytes (Lung)
Cell counts
2500000
2000000
1500000
1000000
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0ppb
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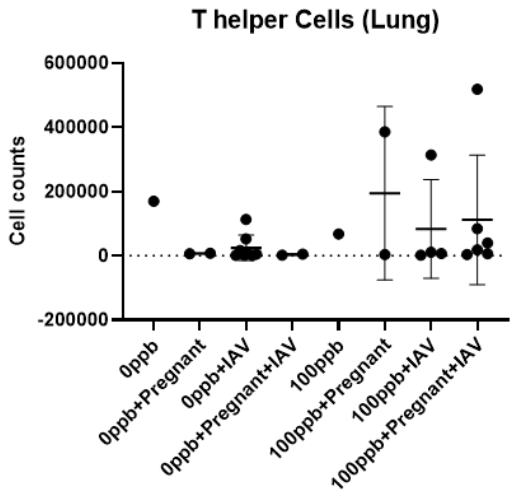
T helper Cells (Lung)
Cell counts
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0
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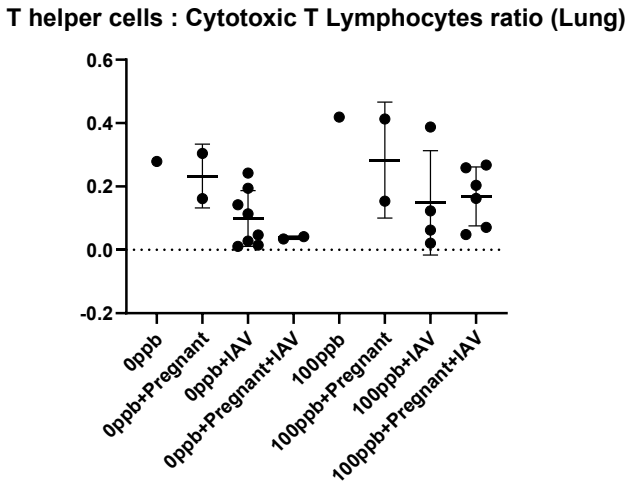
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-0.2
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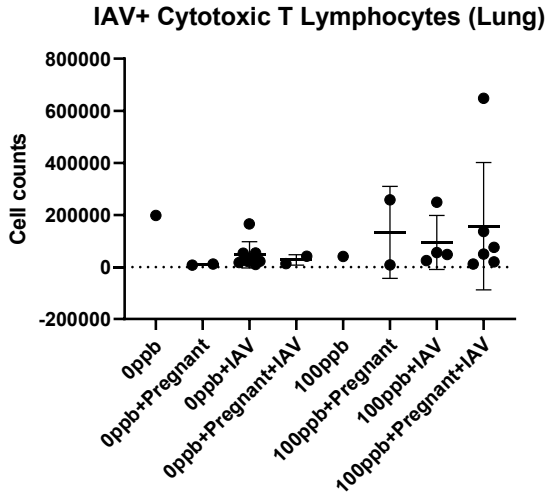
0ppb+Pregnant
0ppb+IAV
0ppb+Pregnant+IAV
100ppb
100ppb+Pregnant
100ppb+IAV
100ppb+Pregnant+IAV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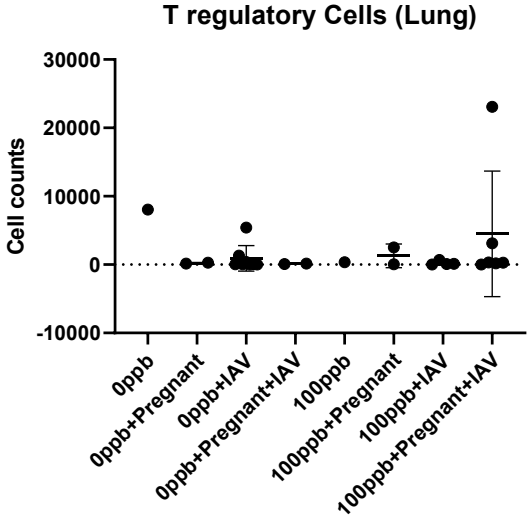
T regulatory Cells (Lung)
Cell counts
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0ppb+Pregnant
0ppb+IAV
0ppb+Pregnant+IAV
100ppb
100ppb+Pregnant
100ppb+IAV
100ppb+Pregnant+IAV

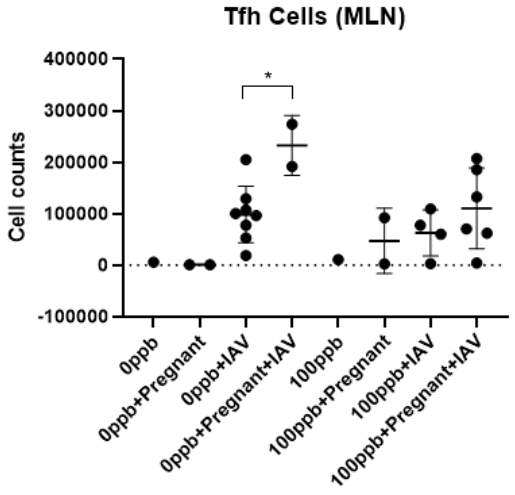
Tfh Cells (MLN)
Cell counts
400000
300000
200000
100000
0
-100000
*
0ppb
0ppb+Pregnant
0ppb+IAV
0ppb+Pregnant+IAV
100ppb
100ppb+Pregnant
100ppb+IAV
100ppb+Pregnant+IAV

Figure 6. Cell counts of different immune cells from the lungs and mediastinal lymph nodes (MLNs).
Mediastinal lymph nodes were assessed for cell counts of B cells, germinal center B cells (GCBC), T follicular helper cells (Tfh), neutrophils, dendritic cells, plasmacytoid dendritic cells, alveolar macrophage, and cytotoxic T lymphocytes. Lungs were assessed for cell counts of neutrophils, dendritic cells, plasmacytoid dendritic cells, alveolar macrophages, T helper cells, cytotoxic T lymphocytes, IAV+ cytotoxic T lymphocytes, and T regulatory cells. Each dot represents data from one animal. 26 mice in eight different treatment groups. No treatment, n=1. Pregnant, n=2. IAV infected, n=8. Pregnant and IAV infected, n=2. 100ppb of arsenic, n=1. 100ppb of arsenic and pregnant, n=2. 100ppb of arsenic and IAV infected, n=4. 100ppb of arsenic, pregnant, and IAV infected, n=6. Samples were collected 8 days after infection (gestation day 18). * *p* value <0.05. IAV: mouse-adapted H1N1 influenza A/California/04/09 (ma2009).

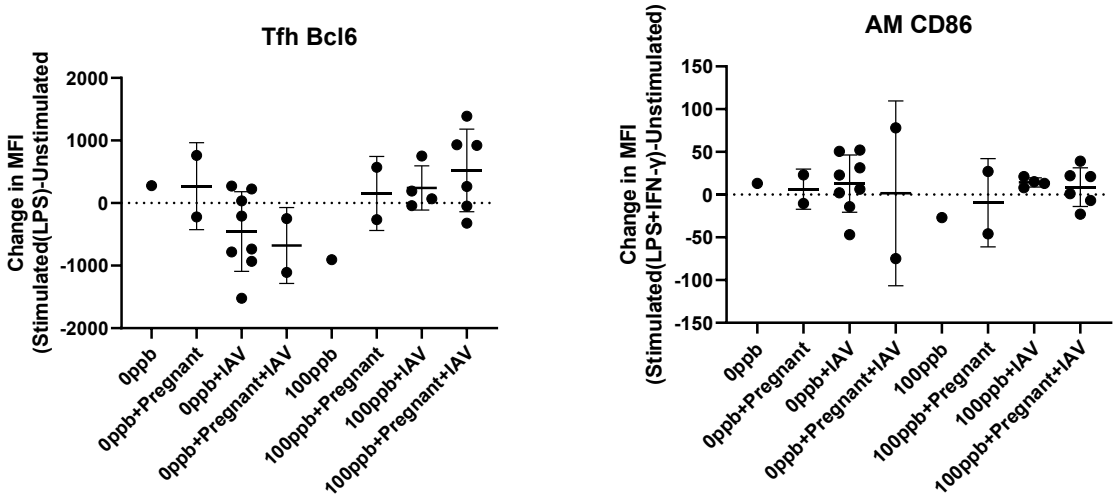

Figure 7. Expression of activation markers and cytokines by different immune cells. Change in mean fluorescent intensity (MFI) after stimulation was assessed for Bcl6 expression in T follicular helper cells (Tfh) in mediastinal lymph nodes, and CD86 surface expression in alveolar macrophages (AM) in lungs. Each dot represents data from one animal. 26 mice in eight different treatment groups. No treatment, n=1. Pregnant, n=2. IAV infected, n=8. Pregnant and IAV infected, n=2. 100ppb of arsenic, n=1. 100ppb of arsenic and pregnant, n=2. 100ppb of arsenic and IAV infected, n=4. 100ppb of arsenic, pregnant, and IAV infected, n=6. Samples were collected on 8 days after infection (gestation day 18). * *p* value <0.05. IAV: mouse-adapted H1N1 influenza A/California/04/09 (ma2009).

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PERSONAL PROFILE

I am a Master of Science Candidate at Johns Hopkins Bloomberg School of Public Health. With my background in environmental health science and biomedical science, I am interested in identifying substances or behaviors that endanger health as well as introducing healthy lifestyles to achieve a better life.

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- Analyzing the effects of arsenic on the immune system during pregnancy and influenza risk

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- Examining the effects of Phytohaemagglutinin and Berberine on Sirt1 inside Jurkat cells

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Student research assistant

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AWARDS

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RESEARCH SKILLS

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Mammalian cell culture: MDCK
Experiments: animal breading, sampling, vaccination, challenge, and euthanasia
Assays: $TCID_{50}$ assay, flow cytometric assay, and cytokine assay
• Statistical software: GraphPad Prism

VOLUNTEER EXPERIENCE

Shin Kong Wu Ho-Su Memorial Hospital, Taipei, Taiwan June 2012 - July 2012

Emergency Room Volunteer